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Calcium-dependent Nedd4-2 upregulation mediates degradation of the cardiac sodium channel

Nav1.5: implications for heart failure

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Abstract

Aim Reductions of voltage-gated sodium channel (Nav1.5) function/expression provide a slowed-conduction substrate for cardiac arrhythmias. Nedd4-2, which is activated by calcium, post-translationally modulates Nav1.5. We aim to investigate whether elevated intracellular calcium ($[Ca^{2+}]_i$) reduces Nav1.5 through Nedd4-2 and its role in heart failure (HF).

Methods Using a combination of biochemical, electrophysiological, cellular and *in vivo* methods, we tested the effect and mechanism of calcium on Nedd4-2 and in turn Nav1.5.

Results Increased $[Ca^{2+}]_i$, following 24-hour ionomycin treatment, decreased sodium current (I_{Na}) density and Nav1.5 protein without altering its mRNA in both neonatal rat cardiomyocytes (NRCMs) and HEK 293 cells stably expressing Nav1.5. The calcium chelator BAPTA-AM restored the reduced Nav1.5 and I_{Na} in NRCMs pretreated by ionomycin. Nav1.5 was decreased by Nedd4-2 transfection and further decreased by 6-hour ionomycin treatment. These effects were not observed in cells transfected with the catalytically inactive mutant, Nedd4-2 C801S or with Y1977A-Nav1.5 mutant containing the impaired Nedd4-2 binding motif. Furthermore, elevated $[Ca^{2+}]_i$ increased Nedd4-2, the interaction between Nedd4-2 and Nav1.5, and Nav1.5 ubiquitination. Nav1.5 protein is decreased whereas Nedd4-2 is increased in volume-overload HF rat hearts, with increased co-localization of Nav1.5 with ubiquitin or Nedd4-2 as indicated by immunofluorescence staining. BAPTA-AM rescued the reduced Nav1.5 protein, I_{Na} and increased Nedd4-2 in hypertrophied NRCMs induced by isoproterenol or angiotensin II.

Conclusion Calcium-mediated increases of Nedd4-2 downregulates Nav1.5 by ubiquitination. Nav1.5 is downregulated and co-localizes with Nedd4-2 and ubiquitin in failing rat heart. These data suggest a role of Nedd4-2 in Nav1.5 downregulation in HF.

Keywords: Arrhythmia · Calcium homeostasis · Heart failure · Nav1.5 · Nedd4-2.

Introduction

The cardiac voltage gated sodium channel, Nav1.5, is responsible for the action potential upstroke and is essential for maintaining an adequate conduction velocity of electrical impulses.^{1, 2} The reduction of Nav1.5 function/expression provides a slowed-conduction substrate for arrhythmias in the setting of

inherited and acquired ion channel disease such as myocardial ischemia and heart failure (HF).³⁻⁵

However, the mechanisms of Nav1.5 downregulation under physiological and pathophysiological conditions requires further investigations.

The activity of Nav1.5 depends on its precise biophysical properties and the density at the plasma membrane which is balanced between forward trafficking and retrograde internalization/degradation.⁶ Nav1.5 activity is further regulated by multiple factors,^{7,8} including the E3 ubiquitin ligase Nedd4-2 (neural precursor cell-expressed developmentally down-regulated 4 type 2), which accelerates the degradation of Nav1.5 by ubiquitination.⁹ Nedd4-2, highly expressed in kidney and heart tissue,¹⁰ where it downregulates target proteins such as epithelial sodium channels (ENaC),¹¹ human ether-a-go-go-related gene (hERG) channel¹² and Nav1.5⁹ through PY (PPxY or LPxY) motifs recognition by its WW domains. However, the regulation of Nedd4-2 activity in cells is poorly understood. Interestingly, the catalytic activity of Nedd4-2 is self-inhibited via intra- or intermolecular C2 and HECT domain interaction. Elevations in intracellular calcium ($[Ca^{2+}]_i$) activate Nedd4-2 through binding of Ca^{2+} to the C2 domain and thereby release the C2 domain-mediated self-inhibition.¹³

Calcium homeostasis is tightly controlled by multiple calcium handling proteins. Mutations or abnormal modifications of these proteins lead to aberrant calcium homeostasis. The altered Ca^{2+} handling in failing hearts, which is characterized by reduced sarcoplasmic reticulum (SR) Ca^{2+} content and increased diastolic cytosolic calcium concentrations due to malfunctioned ryanodine receptor 2 (RyR2) channels, results in systolic and/or diastolic dysfunction and altered downstream signaling.^{14,15} Gene mutations of RyR2 channels, such as in RyR2-P2328S mutant mice, also exhibit increased ventricular cytosolic $[Ca^{2+}]_i$ resulting from RyR2-mediated Ca^{2+} leak.¹⁶ Thus, we hypothesized that increased cytosolic $[Ca^{2+}]_i$ mediated-activation of Nedd4-2 accelerates the degradation of Nav1.5 and leads to

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reduced sodium current (I_{Na}) densities, which may provide a novel possible mechanism of Nav1.5 regulation in heart failure.

Results

Increased $[Ca^{2+}]_i$ downregulates Nav1.5 and I_{Na}

Cells treated with calcium ionophore, ionomycin (IM, 1 μ M, Sigma, USA), to increase $[Ca^{2+}]_i$ (supplementary material Figure S1) were used to examine Nav1.5 expression and sodium current (I_{Na}) density. 24-h IM treatment significantly reduced the total Nav1.5 protein in neonatal rat cardiomyocytes (NRCMs), while 6-h IM treatment did not reduce the total Nav1.5 protein (Figure 1a). Both 6-h and 24-h treatment reduced Nav1.5 expression in the membrane- rich protein fraction ($P<0.05$, Figure 1a), but did not reduce Nav1.5 mRNA level ($P>0.05$, Figure 1c). Both 6-h and 24-h IM treatment reduced the I_{Na} density (Figure 1d, and 1e) of cardiomyocytes without altering the voltage-dependence of steady-state activation and inactivation of the channels remaining at the cell membrane (Figure 1f, see detailed data in supplementary material Table S1). The sarco/endoplasmic reticulum Ca^{2+} ATPase inhibitor thapsigargin (Thap, 1 μ M) which was used to increase diastolic $[Ca^{2+}]_i$,^{17, 18} also reduced Nav1.5 protein but not its mRNA. The calcium chelator BAPTA-AM (BAP, 5 μ M) restored the reduced Nav1.5 protein in NRCMs pretreated with IM or thapsigargin comparable to a normal level (Figure 1g). BAP also reversed the reduced I_{Na} pre-incubated with IM in NRCMs (Figure 1h). In parallel, 6-h IM treatment in Nav1.5-HEK cells exerted no effects on Nav1.5 in total protein lysates, while 24-h treatment caused a reduction of approximately 40%. Both 6-h and 24-h treatment caused a decrease of approximately 60% of Nav1.5 in the membrane- rich protein fraction ($P<0.05$, Figure 2a) compared

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with the control (Ctrl), whereas it did not reduce Nav1.5 mRNA level (*Figure 2c*). Correspondingly, the peak I_{Na} density was reduced by approximately 60% after IM treatment for 6 h (I_{Na} density: IM 6 h -134.05 ± 16.74 vs. Ctrl -318.06 ± 36.49 pA/pF, $P < 0.01$) and 24 h (I_{Na} density: IM 24h -153.30 ± 13.76 vs. Ctrl -318.06 ± 36.49 pA/pF, $P < 0.01$; *Figure 2d, e, f*). BAP also rescued the reduced I_{Na} pretreated with IM (*supplementary material Figure S2*). The voltage-dependent steady-state activation and inactivation (*Figure 2g*) as well as the recovery from fast inactivation (*Figure 2h*) were not significantly altered by IM treatment (*the detailed parameters are given in supplementary material Table S2*). These results suggest that the reduced I_{Na} density induced by ionomycin results from a decreased number of functional channels at the plasma membrane. To test the specificity of IM on Nav1.5, other ion channels were also detected. It has been reported that in adult ventricular myocytes, transient changes in $[Ca^{2+}]_i$ can modulate Cav1.2 mRNA and protein abundance.^{19, 20} In the present study, IM reduced the protein level of Cav1.2 in NRCMs. KCNQ1 possesses a PY motif in the intracellular C-terminal domain. Previous study shows that KCNQ1 proteins expressed in HEK 293 are reduced by Nedd4-2.¹⁰ In the present study, IM treatment also reduced KCNQ1 protein in NRCMs. Kv4.3, the ion channel that mediate the transient outward potassium current, and Kv1.5, the ion channel that confer the ultra-rapid delayed-rectifier potassium channel currents, were not reduced in NRCMs treated by IM (*see detailed data in supplementary material Figure S3*).

Nedd4-2 reduces Nav1.5 protein level and I_{Na}

A GFP-Nav1.5 construct was transiently transfected into HEK 293 cells along with Nedd4-2 WT, Nedd4-2 CS or empty pcDNA3 vector to record I_{Na} (Figure 3). WT Nedd4-2 decreased the peak I_{Na} density by approximately 42% compared with the empty vector (I_{Na} density: pcDNA3 -378.55±57.60 vs. Nedd4-2 -218.39±34.57 pA/pF, $P<0.05$; Figure 3b), whereas Nedd4-2 CS did not significantly affect the I_{Na} density (I_{Na} density: pcDNA3 -378.55±57.60 vs. Nedd4-2 CS -315.53±39.59 pA/pF, $P>0.05$; Figure 3b). In addition, Nedd4-2 co-transfection reduced Nav1.5 expression level by approximately 40% ($P<0.05$, Figure 3d), while Nedd4-2 CS co-transfection did not significantly reduce Nav1.5 expression ($P>0.05$, Figure 3d). Co-IPs were used to further test the interaction between these two proteins. HEK 293 cells were co-transfected with Nav1.5 and Nedd4-2 or Nedd4-2 CS plasmids together with incubation of proteinase inhibitor MG132 for 24 hours. Then the cells were harvested and immunoprecipitated by anti-Nav1.5 antibody and the precipitated fractions were immunoblotted using anti-Nav1.5 and anti-Nedd4-2 antibodies respectively. The ratio of Nedd4-2/Nav1.5 represent the bound efficiency between these two proteins. The results show that Nedd4-2 bound to Nav1.5 much more efficiently than Nedd4-2 CS did (Figure 3e and 3f, $P<0.01$).

Elevated $[Ca^{2+}]_i$ increases the expression and function of Nedd4-2

Acute increases in $[Ca^{2+}]_i$ were recently shown to activate endogenous Nedd4-2 activity by binding to the C2-domain of Nedd4-2 in HEK 293T cells.¹³ In our study, as shown in Figure 4a and 4b, elevations in $[Ca^{2+}]_i$ by IM significantly increased the endogenous Nedd4-2 protein level ($P<0.05$) in Nav1.5-HEK cells. Similar results were obtained in NRCMs treated either with IM ($P<0.05$) or thapsigargin ($P<0.05$).

Both IM and thapsigargin increased Nedd4-2 mRNA level in NRCMs (*Figure 4c*). The Co-IP assay was used to investigate the role of calcium on Nedd4-2 and Nav1.5 interactions as well as Nav1.5 ubiquitination level. HEK 293 cells transfected with Nav1.5 together with pcDNA3, Nedd4-2 or Nedd4-2 CS were treated without (Ctrl) or with IM in the presence of MG132 (proteasome inhibitor). As shown in *Figure 4d* and *4e*, IM treatment robustly increased Nedd4-2/Nav1.5 ratio in Nedd4-2 or Nedd4-2 CS transfected cells, immunoprecipitated by anti-Nav1.5 antibody, and the Nav1.5/Nedd4-2 ratio, immunoprecipitated by anti-HA tag antibody. In addition, Nav1.5 ubiquitination level under IM treatment was further tested. HEK 293 cells transiently transfected with Nav1.5-WT together with Nedd4-2 or Nedd4-2 CS were immunoprecipitated (IP) by anti-ubiquitin antibody and then immunoblotted (IB) with anti-Nav1.5 antibody to test the ubiquitinated Nav1.5. As shown in *Figure 4f*, compared with Nedd4-2 WT co-transfection, a robust decrease in incorporated ubiquitin was observed in Nedd4-2 CS co-transfected cells ($^{\#}P<0.05$ vs. Nedd4-2), and IM treatment significantly increased Nav1.5 ubiquitination level in Nedd4-2 but not in Nedd4-2 CS co-transfected cell ($^{*}P<0.05$ vs. Nedd4-2). These results suggest that elevated $[Ca^{2+}]_i$ increases Nedd4-2 expression, the interaction between Nav1.5 and Nedd4-2, and consequently increases Nav1.5 ubiquitination.

Calcium downregulates Nav1.5 via Nedd4-2 pathway

Nav1.5-HEK cells were transfected either with Nedd4-2, Nedd4-2 CS or pcDNA3 for 24 h and then treated without (Ctrl) or with IM for 6 h to increase $[Ca^{2+}]_i$. In line with the previous results in *Figure 2a*, as illustrated in *Figure 5a*, Nav1.5 expression was not significantly reduced by 6-h IM treatment in pcDNA3-transfected ($P>0.05$) or Nedd4-2 CS-transfected cells ($P>0.05$), but was significantly reduced

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in Nedd4-2-transfected cells ($P<0.05$). The previous studies demonstrated that Y1977A mutation disrupts the conserved amino acids of PY motif (PPxY) in Nav1.5 and abolished the interaction with Nedd4-2 WW domain. Consequently, the expression and function of Y1977A-Nav1.5 were not affected by Nedd4-2.^{9, 21} To further assess whether IM decreased Nav1.5 was dependent on Nedd4-2-Nav1.5 interaction, the Y1977A-Nav1.5 mutant was constructed. As anticipated, 24-hour IM treatment reduced Nav1.5 in HEK 293 cells transiently transfected with WT-Nav1.5 but not with Y1977A-Nav1.5 mutant (Figure 5c, 5d). In addition, the Y1977A mutation abolished the Nedd4-2 overexpression-mediated decrease in Nav1.5 expression, and also abolished IM treatment-mediated Nav1.5 reduction under Nedd4-2 transfection (Figure 5e, 5f).

Nav1.5 downregulation correlates with increased Nedd4-2 expression and accompanied by increased Nav1.5 and Nedd4-2 / ubiquitin co-localization in HF

Rats in the HF group showed obvious clinical signs of HF, such as tachypnea, appetite loss, and lethargy. Twelve weeks after the operation, all HF rats had a significant decrease in the percentage of ejection fraction (EF) and fractional shortening (FS), and also had significant ventricular dilation compared with the SO rats based on the echocardiographic data (supplementary material Table S3), indicating successful construction of the model. Figure 6a and b show that Nav1.5 expression declined markedly ($P<0.05$), while Nedd4-2 expression was significantly increased ($P<0.01$), in the HF group (n=6) compared with the SO group (n=6). There is no significant difference in Nav1.5 mRNA level between HF and SO rat heart, whereas the Nedd4-2 mRNA was increased in the failing rat heart (Figure 6c). Since Nedd4-2 is an E3 ligase that binds to target protein substrates, transferring ubiquitin from an

E2 conjugating enzyme to the substrate, we investigated the co-localization of Nav1.5 and Nedd4-2/ubiquitin to elucidate the mechanism underlying Nav1.5 downregulation in HF. *Figure 6d* shows that Nav1.5 was distributed in a striated pattern, which is similar to the previous study,⁴ while Nedd4-2 was distributed throughout the whole cell. The Nedd4-2 expression and co-localization with Nav1.5 were increased in HF relative to SO rats, especially in the plasma membrane of adult rat cardiomyocytes. *Figure 6e* shows that ubiquitin was distributed throughout the cell and that its co-localization with Nav1.5 was also increased in HF. No signal above background was detected in negative-control cardiomyocytes (data are not shown).

BAPTA-AM rescues Nav1.5 in hypertrophied NRCMs induced by isoproterenol or angiotensin II

NRCMs treated with isoproterenol (ISO, 10 μ M) or angiotensin II (AngII, 1 μ M) for 48 h was used to mimic the over-activation of the neuroendocrine system in heart failure.^{22, 23} And their effects on calcium homeostasis were studied by confocal image analysis. Similarly to the previous studies,^{24, 25} we also found that AngII treatment increased systolic and diastolic $[Ca^{2+}]_i$ but impaired Ca^{2+} transient amplitudes, chronic isoproterenol treatment increased the peak amplitude of calcium transients and the $[Ca^{2+}]_i$ in diastolic phase. BAP reduced the increased $[Ca^{2+}]_i$ in systolic and diastolic phase induced both by ISO and AngII (see *detailed data in supplementary material Figure S4*). Similar to the failing hearts, both ISO and AngII significantly reduced Nav1.5 and increased Nedd4-2, and these effects were restored by the presence of BAP (*Figure 7a, b*). The functional role of ISO on Nav1.5 was further analyzed by whole-cell patch clamp. BAP restored the macroscopic I_{Na} (*Figure 7c*) and peak I_{Na} density (*Figure 7d*) decreased by ISO. ISO did not alter the gating properties of I_{Na} in NRCMs. Also, BAP did

not significantly affect the voltage-dependent inactivation, the half maximal voltage of the steady-state activation ($V_{1/2}$), or the slope factor (k) (Figure 7e and 7f, detailed data are given in supplementary material Table S4).

Discussion

The present study identifies a novel mechanism in which calcium reduces Nav1.5 expression and function through Nedd4-2 mediated ubiquitination. The major findings are: (1) Elevated $[Ca^{2+}]_i$ increased Nedd4-2 expression and function, in turn accelerating the degradation of Nav1.5 and, as a result, downregulated the density of Nav1.5 at the plasma membrane. (2) The expression of Nedd4-2 and its co-localization with Nav1.5 was increased in failing rat hearts, which was correlated with increased ubiquitination of Nav1.5 channels.

Increased $[Ca^{2+}]_i$ reduces Nav1.5 expression via multiple mechanisms

The life cycle of Nav1.5 starts from the transcription of the SCN5A gene, mRNA processing, protein synthesis and modifications, and followed by trafficking to the plasma membrane, where it exerts its functional role, finally endocytosis from the membrane to be degraded or recycled.¹ Factors that affect any stage in this life path could result in altered Nav1.5 expression and function. A few reports have indicated that Nav1.5 mRNA and protein levels are downregulated by the calcium ionophores A23817 in neonatal rat cardiomyocytes assessed by Northern blot hybridization.^{26,27} Whereas, in the present study, both ionomycin and thapsigargin did not reduce Nav1.5 mRNA level, as indicated by RT-qPCR.

This discrepancy may be due to different methods used to increase calcium concentration and mRNA detection technique. In another recent study, I_{Na} in cardiomyocytes was decreased by acutely increasing $[Ca^{2+}]_i$ in the internal pipette solution in a concentration-dependent manner without modifying I_{Na} gating properties,²⁸ but the mechanism is still unknown. In our case, we found that elevations of $[Ca^{2+}]_i$ by 24-hour ionomycin significantly reduced Nav1.5 protein and I_{Na} both in NRCMs and HEK 293 cells. Furthermore, IM treatment decreased I_{Na} and reduced Nav1.5 protein in the membrane-rich fraction to a greater extent than in whole-cell lysates, which suggested that calcium was able to reduce Nav1.5 membrane density. These data further indicated that besides the transcriptional regulation of Nav1.5, there exist other mechanisms underlying calcium-mediated Nav1.5 reductions.

Calcium reduces Nav1.5 protein via enhancing Nedd4-2 function

Ca^{2+} binding to the C2-domain of Nedd4-2, upon increased $[Ca^{2+}]_i$, enables its translocation to the cell membrane, resulting in dissociation of the auto-inhibitory C2 domain from the HECT domain, and thus activates Nedd4 E3 ligase activity in HEK 293T cells.¹³ Our studies demonstrated that elevation of $[Ca^{2+}]_i$ not only increased Nedd4-2 function, which was reflected by increased Nav1.5 ubiquitination, but also up-regulated the endogenous Nedd4-2 protein and mRNA level in NRCMs. There is lack of effect of IM treatment on Nedd4-2 total protein expression in HEK 293 cells transfected with the CMV-controlled Nedd4-2 transcription plasmid as it is illustrated in *Figure 5a and 5e*. These results would suggest that the effect of ionomycin in NRCMs could be related to increased Nedd4-2 transcription, which is not observed in transfected cells because Nedd4-2 expression is controlled from an exogenous promoter. Calcium has been recognized as one of the most versatile second messengers,²⁹ which

regulates expression of various genes by activation of transcription factors, including myocyte enhancer factor 2 (MEF2), GATA4 or nuclear factor of activated T cells (NFAT) in cardiomyocytes.^{30,31}

However, till now little is known about the transcription regulation of Nedd4-2, further work will be required to determine whether the increased Nedd4-2 expression may result from calcium activated transcription factors. Nedd4-2 ubiquitinates Nav1.5, leading to its internalization/degradation and thus reducing Na⁺ currents without affecting forward trafficking or modifying the biophysical properties of the channel.^{9,21} In our case, the increased interaction between Nedd4-2 and Nav1.5 likely result from both increased Nedd4-2 expression and calcium mediated Nedd4-2 activation reported by wang et al.¹³

6-hour IM treatment decreased the membrane density of Nav1.5 but had no significant effect on the total expression of Nav1.5, indicating that prior to the complete degradation, the ubiquitinated Nav1.5 may be redistributed from the plasma membrane to the intracellular pool. Our data further showed that Nav1.5 protein was decreased by 6-hour IM treatment in Nedd4-2- co-transfected but not in Nedd4-2 CS- or pcDNA3- co-transfected Nav1.5-HEK cells. Correspondingly, the ubiquitinated Nav1.5 level in Nav1.5-HEK cells was increased by IM treatment in Nedd4-2- but not in Nedd4-2 CS- co-transfected cells. Nedd4-2 was demonstrated to reduce Nav1.5 membrane density by binding to the PY-motif (PPxY) in Nav1.5. Y1977A mutation in Nav1.5 disrupt the PY motif and abolished the effects of Nedd4-2 on Nav1.5.^{9,21} In our results, IM treatment exerts no effect on Y1977A-Nav1.5 in presence or absence of Nedd4-2 co-transfection. These results demonstrate that calcium reduces Nav1.5 expression by enhancing Nedd4-2 function.

Calcium-activated Nedd4-2 mediating the downregulation of Nav1.5 may provide a novel possible mechanism of Nav1.5 regulation in heart failure

Previous studies demonstrated that Nav1.5 expression is decreased in HF.^{3,4} Zicha S *et al.* reported that the peak sodium currents are significantly reduced in HF dog models and in human failing cardiomyocytes despite no differences in Nav1.5 mRNA levels,³² suggesting that Nav1.5 is post-transcriptionally regulated. Mechanisms including the expression of non-functional Nav1.5 splicing variants^{23,33} or probably the phosphorylation by Ca²⁺/CaM-dependent protein kinase II (CaMKII).³⁴ In the Dudley group, they found that the full length of SCN5A transcript E28A was decreased by 24.7% in HF, however the Western Blot analysis revealed a 62.8% Nav1.5 protein reduction in HF,³⁵ indicating that Nav1.5 is regulated at the protein level in the failing heart. In our results, Nav1.5 protein is decreased whereas Nav1.5 mRNA level were not reduced in the failing heart. Nedd4-2 is increased in HF accompanied by increased co-localization of Nedd4-2 and Nav1.5 heart failure by immunofluorescent assay. These data suggest that Nedd4-2 may play a role in Nav1.5 downregulation in heart failure. The failing heart is presented by increased diastolic calcium concentrations, and elevations of [Ca²⁺]_i by IM or thapsigargin increased Nedd4-2 expression and function, which suggests that the increased Nedd4-2 in HF hearts results from long-term alterations of calcium homeostasis. Previous studies demonstrated that Nedd4-2 interacts with Nav1.5 via *in vitro* pull-down and yeast two-hybrid assays, but failed to co-immunoprecipitate these proteins due to their low binding affinity.²¹ In the present study, although we failed to co-immunoprecipitate Nav1.5 with endogenous Nedd4-2 in heart tissue, the interaction was observed after these two proteins were over-expressed in HEK 293 cells and was indirectly reflected by immunofluorescent co-localization in isolated cardiomyocytes. Increased co-localization of Nav1.5 and ubiquitin, suggest a possibility that Nedd4-2-mediated ubiquitination

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probably participated in Nav1.5 downregulation in HF. However, we should still acknowledge that the interaction between Nav1.5 and Nedd4-2/ ubiquitin indicated by immunofluorescent co-localization could be further validated by use of knock-out animals to further verify the specificity of the staining.

In vitro studies show that elevation of $[Ca^{2+}]_i$ reduced Nav1.5 protein and sodium current, and decreasing $[Ca^{2+}]_i$ by BAPTA-AM^{36,37} not only restored the reduced Nav1.5 expression in hypertrophied NRCMs induced by ISO or AngII, but also increased I_{Na} density. ISO and AngII were used to treat NRCMs to mimic the over-activation of sympathetic nervous system and rennin-angiotensin- aldosterone system in heart failure. AngII was reported to reduce I_{Na} through H_2O_2 mediated transcription reduction,³⁸ and by activation of splice factor RBM25 and LUC7L3.^{23,39} While another report showed that the chronic ANGII type 1 receptor activation reduced I_{Na} density by about 60%, but it could not be explained by the slightly reduced Nav1.5 mRNA level.³⁹ These data suggest that different mechanisms are involved in AngII mediated downregulation of I_{Na} . Several studies have indicated that acute exposure to ISO in the bath solution enhanced Na^+ conductance.^{40,41} In a recent study, mouse cardiomyocytes exposed to ISO acutely reduced the membrane distribution of Nav1.5, but increased the peak I_{Na} by negatively shifting I_{Na} activation.⁴² However, the chronic effects of ISO on Nav1.5 were unclear. We have now found that the chronic ISO and Ang II treatment decrease Nav1.5 and increase Nedd4-2 expression in NRCMs. These effects can be reversed by the calcium chelator BAP. Both ISO and Ang II increased diastolic and systolic $[Ca^{2+}]_i$ in NRCMs, and the increased $[Ca^{2+}]$ can be reduced by BAP (see detailed data supplementary material *Figure S4*). These data suggest a possibility that calcium-mediated expression of Nedd4-2 may participate in Nav1.5 downregulation in the hypertrophied cardiomyocytes. While caution must be taken when using experimental data obtained in cultured cells to interpret observations *in vivo* animals, our data suggest a possibility that the altered

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calcium homeostasis in heart failure may decrease Nav1.5 via increasing Nedd4-2 expression. Further studies using the Nedd4-2 knock out/ down animal model are needed to establish a direct mechanistic link.

In conclusion, our work demonstrates that calcium increases Nedd4-2 expression and enhances the interaction between Nedd4-2 and Nav1.5, consequently reducing Nav1.5 membrane density via Nedd4-2-mediated ubiquitination (as illustrated in *Figure 8*). Nav1.5 is downregulated and accompanied by increased co-localization with Nedd4-2 and ubiquitin in a volume-overload HF model. These observations suggest a role of Nedd4-2 in Nav1.5 regulation in heart failure.

Limitations

This study has two main limitations: 1) The altered calcium handling in HF is very complicated, and could not be completely mimic by *in vivo* experiment models. We use ionomycin or thapsigargin to increase or BAPTA-AM to decrease calcium concentration, and those are different from HF and may activate other cellular process. 2) *In vivo* studies of the functional role of Nedd4-2-mediated downregulation of Nav1.5 in HF using Nedd4-2 knockout models are needed. Drugs that remedy the altered calcium homeostasis in HF should also be used to test the effect of calcium on Nedd4-2 expression *in vivo*.

Materials and Methods

In vivo animal studies

(1) Volume-overload HF model. Male Sprague-Dawley rats (weighing 140-160 g) were obtained from the Laboratorial Animal Center of Xi'an Jiaotong University (Xi'an, Shaanxi, China) and randomly assigned to two groups, sham operation (SO) and HF. The volume-overload HF model was established as reported previously with some modifications.^{43, 44} Briefly, the rats were anesthetized by intraperitoneal injection of sodium pentobarbital (40-45 mg/kg body weight). The depth of anesthesia was monitored by the absence of pedal withdrawal upon toe pinching and the loss of corneal reflex. The abdominal cavity was opened along the linea alba (about 4 cm), the shunts were achieved by puncturing the aorta with vena cava. The abdominal incision was sutured and the rats were allowed to recover. Rats were given buprenorphine (0.1 mg/kg) subcutaneously every 8-12 h for the initial 24 h for analgesia and minimizing pain caused by surgical operations. The rats were then maintained in cages at 21±1 °C with 12 h light/dark cycles. Rats in the SO group had a similar operation without establishing a fistula. When the volume-overload rat HF model was successfully established, rats were euthanized by pentobarbital sodium (200 mg/kg, i.p.) and the hearts were excised for further analysis. All procedures were approved by the Care of Experimental Animals Committee of Xi'an Jiaotong University, and the investigation conforms to the US National Institutes of Health guidelines (Guide for the Care and Use of Laboratory Animals).

(2) Echocardiography. Twelve weeks post surgery, the rats were anesthetized by inhalation of 2 % isoflurane, placed in a supine position on a hotplate. Then the transthoracic echocardiography was performed to monitor the thickness of left ventricular wall, the systolic and diastolic cardiac function

using an iE33 Imaging System (Philips Healthcare, Andover, MA) equipped with a 30-MHz microprobe.⁴⁵

(3) Isolation of adult rat cardiomyocytes. Single cardiomyocytes were isolated as described previously.⁴⁴ Briefly, the rats were anaesthetized with sodium pentobarbital (40-45 mg/kg, i.p) and anti-coagulated with heparin sodium (400 IU/ kg, i.p.). The hearts were quickly excised by thoracotomy, cannulated via a Langendorff perfusion apparatus, and then digested using 179 IU/mL collagenase type I (Sigma, USA), 0.01 % protease (Sigma, USA) and 0.06% bovine serum albumin dissolved in Tyrode's solution at 37 °C. The isolated cardiomyocytes were stored in Kraftbrühe (KB) solution at room temperature (22-24 °C). At least 80 % of the isolated cardiomyocytes were rod shaped with clear striation.

(4) Immunofluorescence staining. The isolated cardiomyocytes were fixed with 4 % paraformaldehyde, permeabilized by 0.1 % Triton X-100 and blocked with 5 % BSA. Goat anti-Nav1.5 and rabbit anti-ubiquitin antibodies (Santa Cruz, USA), rabbit anti-Nedd4-2 (Cell Signaling Technology, USA), donkey anti-rabbit Alexa Fluor 555-labeled and donkey anti-goat Alexa Fluor 488-labeled secondary antibodies (Life Technologies, USA) were used for immunofluorescence assays. Images of immunostained cardiomyocytes were captured on a TCS laser scanning confocal microscope (Leica, Germany). Negative control cells were incubated with secondary antibodies without primary antibodies.

In vitro cell studies

(1) Cell culture and plasmid transfection. Human Flag-tagged Nav1.5 (hH1c, FLAG was inserted into the domain I extracellular S1–S2, between Pro 153 and Pro 154) cDNA in pcDNA3 was a kind gift from Dr. Yutao Xi (Texas Heart Institute, USA), the human Nav1.5 cDNA in pEGFP-N2 expression vector

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was a kind gift from Dr. Yanmin Zhang (University of Manchester, UK). It has been shown that neither the Flag- nor GFP-tag affects the function of the Nav1.5 channel. The open reading frame of human Nedd4-2 cDNA (KIAA0439, contains C2-domain coding sequence) in pBluescript II plasmid (Kazusa DNA Research Institute, Japan) was amplified using polymerase chain reaction (PCR) and cloned into HA-pcDNA3 (Invitrogen) to generate HA-tagged Nedd4-2. The catalytic inactive form of Nedd4-2, Nedd4-2 C801S mutant (Nedd4-2 CS, Cys⁸⁰¹ mutated to Ser) and Nav1.5 with disrupted PY motif (PPxY), Y1977A-Nav1.5 mutant were generated using site-directed PCR mutagenesis and verified by sequencing. HEK 293 cells were maintained in Dulbecco's modified Eagle's medium (DMEM, Hyclone) with 10% fetal bovine serum (Gibco, USA) at 37 °C in 5% CO₂. Lipofectamine 3000 (Invitrogen, CA, USA) was used for plasmid transfection into HEK 293 cells. HEK 293 cells stably expressing Nav1.5 (Nav1.5-HEK cells) were maintained via G418 (0.4 mg/mL, Sigma, USA) and verified by Western Blot and patch clamp assay.

(2) Neonatal rat cardiomyocytes isolation. Neonatal rat cardiomyocytes (NRCMs) were isolated according to the procedures described elsewhere.⁴⁶

(3) Real-Time qPCR and Western blot analysis. Total RNA was Trizol-extracted (Takara, Japan), and 500 ng RNA was reverse transcribed into cDNA using the reverse transcription kit (Takara, Japan). All quantitative PCR analyses were performed using a SYBR[®] Premix Ex Taq[™] II (Takara, Japan) following manufacturers' protocol. RT-qPCR were analyzed by $\Delta\Delta$ Ct method, normalized to GAPDH and represented relative the control.

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Proteins from whole-cell or membrane-rich fraction were extracted using RIPA buffer or the Mem-PER Eukaryotic Membrane Protein Extraction Reagent Kit (Thermo Scientific, USA) respectively, and separated by 8 % SDS-PAGE, and transferred to PVDF membranes (Millipore, USA). The membranes were probed with either anti-Nav1.5 (Alomone, Israel), Cav1.2 (Alomone, Israel), KCNQ1 (Santa Cruz, USA), Kv1.5 (Santa Cruz, USA), Kv4.3 (Santa Cruz, USA), or anti-Nedd4-2 (Cell Signaling Technology, USA) antibody, followed by horseradish peroxidase-conjugated secondary antibody (goat anti-rabbit IgG-HRP, Santa Cruz, USA). Finally, the proteins were detected using ECL (Bio-Rad, USA) on a chemiluminescence system (ChemiDoc XRS, Bio-Rad, USA). Actin (antibody from Santa Cruz, USA) was used for protein normalization. All band intensities were quantified by densitometry.

(4) Co-immunoprecipitation (Co-IP) analysis. For Co-IP detection, 0.5 mg of protein in 0.5 mL lysis buffer was incubated with either anti-Nav1.5 (Santa Cruz, USA), anti-HA (Sino Biological Inc., China), anti-ubiquitin (Sigma, USA) or anti-GAPDH (Santa Cruz, USA) antibodies at 4 °C for 4 h, followed by overnight incubation with protein A/G Plus-agarose beads (Santa Cruz, USA) at 4 °C on a rotator. After, they were washed three times with RIPA buffer, the immunoprecipitates were collected for Western blot analysis.

(5) Electrophysiological recordings. Sodium currents were recorded using whole-cell voltage-clamp techniques (MultiClamp 700B amplifier, Axon Instruments Inc., USA) at room temperature (22-24 °C).

For I_{Na} recordings in Nav1.5 HEK cells, the pipette solution contained (mM): NaCl 10, CsCl 130, HEPES 10, and EGTA 10 (pH 7.3 with CsOH), and the bath solution contained (mM): NaCl 110, KCl 4, MgCl₂ 1, CaCl₂ 1.8, HEPES 10 and glucose 10 (pH 7.3 with NaOH). For I_{Na} recordings in NRCMs, the bath solution contained (mM): NaCl 20, tetraethylammonium (TEA) chloride 115, CsCl 5, MgCl₂ 1, HEPES 10 and glucose 10 (pH 7.35 with NaOH) and the pipette solution contained (mM): NaCl 5, CsF

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115, CsCl 20, HEPES 10, and EGTA 10 (pH 7.3 with CsOH). Currents were digitized at a sampling rate of 10 kHz and filtered at 2 or 5 kHz (Digidata 1440A, Axon Instruments Inc., USA). I_{Na} was recorded by 20 ms step depolarizations to different potentials between -80 and +60 mV from a holding potential of -140 mV. The voltage-dependence of steady-state inactivation was obtained by a 25-ms test pulse to -10mV after a pre-pulse of 500-ms at different membrane potentials (V_m). Recovery from inactivation was analyzed using the double-pulse protocol at 0.1 Hz. From a holding potential of -90 mV, the membrane was first pulsed to -30 mV for 1 s to induce inactivation (P1). The membrane was then repolarized to -90 mV for varying periods before the second pulse to -30 mV for 50 ms was applied. The current amplitudes upon P2 were plotted against P1-P2 intervals. Data were analyzed using Clampfit 10.2 (Axon Instruments Inc., USA).

Statistical analysis

All data are presented as the mean \pm standard error of the mean. Two-tailed independent Student's *t* tests were used to test the statistical significance between two groups. One-way analysis of variance was used for multiple comparisons with the Bonferroni test for post hoc testing. The null hypothesis was rejected for $P < 0.05$.

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Conflict of interests

The authors declare that there is no conflict of interests in relation to the publication of this study.

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Figure legends

Figure 1 Elevated $[Ca^{2+}]_i$ decreased Nav1.5 protein and I_{Na} in NRCMs. NRCMs were treated with ionomycin (IM, 1 μ M) or thapsigargin (Thap, 1 μ M) to increase $[Ca^{2+}]_i$. (a), Representative immunoblot and (b) densitometric measurements (normalized to actin) of Nav1.5 in total protein and membrane protein extracts in NRCMs treated without (Ctrl) or with IM for 6 h or 24 h. $n=3$, $*P<0.05$ vs. Ctrl. (c) The SCN5A mRNA level in NRCMs (normalized to GAPDH). (d), Typical I_{Na} current traces in NRCMs cells treated without IM (Ctrl) or with IM for 6 h (IM 6h) or 24 h (IM 24h). (e), The I/V curve of the summarized I_{Na} . $n=10\sim14$ cells, $*P<0.05$ vs. Ctrl. (f), Steady-state activation and inactivation curves. Activation properties were determined from I/V relationships, and the normalized conductance was plotted against membrane voltage. Inactivation properties were estimated by normalized peak currents plotted against membrane voltage. Steady-state activation and inactivation curves were fitted with the Boltzmann equation. NRCMs were pre-treated with IM or Thap for 6 h, then BAPTA-AM (BAP, 5 μ M) was added for further incubation of 24 h. Then the cells were harvested for western blot analysis to detect Nav1.5 protein level or for patch clamp study to record I_{Na} . (g) The densitometric measurements of Nav1.5 to actin were presented below the representative immunoblot ($n=3$, $*P<0.05$ vs. Ctrl, $^{\#}P<0.05$ vs. Thap, $^{\$}P<0.05$ vs. IM). (h) The I/V curve of the summarized I_{Na} in NRCMs pretreated with IM or IM+BAP as indicated. $n=12\sim18$ cells, $*P<0.05$ vs. Ctrl, $^{\#}P<0.05$ vs. IM.

Figure 2 Ionomycin decreased Nav1.5 protein and I_{Na} in Nav1.5-HEK cells. Nav1.5-HEK cells were treated without (control, Ctrl) or with ionomycin (IM, 1 μ M) to increase $[Ca^{2+}]_i$ for 6 h or 24 h, then the cell were harvested for further analysis. (a), Representative immunoblot and (b) densitometric

measurements (normalized to actin) of Nav1.5 in total protein and membrane protein extracts of Nav1.5-HEK cells. n=3, * $P<0.05$ vs. Ctrl. (c), The SCN5A mRNA level in Nav1.5-HEK cells (normalized to GAPDH). (d), Typical I_{Na} current traces in Nav1.5-HEK cells treated with IM for 6 h (IM 6h) and 24 h (IM 24h) or without IM (control, Ctrl). (e), The summarized peak I_{Na} density. n=12~13 cells, $\dagger P<0.01$ vs. Ctrl. (f), The I/V curve of the summarized I_{Na} . (g), Steady-state activation and inactivation curves. The data were obtained and analyzed using same protocols and equations used in Figure 1f. (h), Recovery curve of I_{Na} was fitted with mono-exponential functions ($I/I_{max}=1 - e^{-\tau/a}$).

Figure 3 Nedd4-2-WT, but not Nedd4-2 CS, interacts with Nav1.5 and decreases Nav1.5 in HEK 293 cells expressing Nav1.5. (a), Representative I_{Na} current traces were obtained from HEK 293 cells co-transfected with GFP-SCN5A plus pcDNA3, Nedd4-2-WT or Nedd4-2 CS. (b), The peak I_{Na} density is summarized in the bar graphs. n=6~10 cells, * $P<0.05$ vs. pcDNA3. Representative immunoblot (c) and (d) densitometric measurements (normalized to actin) of Nav1.5 in Nav1.5-HEK cells transfected with pcDNA3, Nedd4-2-WT or Nedd4-2 CS, n=3, * $P<0.05$ vs. pcDNA3. HEK 293 cells were transfected with Nav1.5 and Nedd4-2 or Nedd4-2 CS plasmids together with MG132 incubation for 24 hours. The cell lysates were then immunoprecipitated (IP) with anti-Nav1.5 or anti-GAPDH (as a negative control) antibody to precipitate Nav1.5 and its associated proteins, then the precipitated fractions were separately immunoblotted (IB) using anti-Nav1.5 and anti-Nedd4-2 antibody as indicated. Input of the precipitation experiments is presented as a control of Nav1.5 and Nedd4-2 expression. The representative immunoblot (e) and (f) densitometric measurements (Nedd4-2/ Nav1.5 ratio) of Nav1.5 and Nedd4-2, n=3, $\dagger P<0.01$ vs. Nedd4-2.

Figure 4 Calcium increased Nedd4-2 expression, Nedd4-2 and Nav1.5 interaction, and Nav1.5 ubiquitination level. (a), Representative immunoblots and (b) densitometric measurements (normalized to actin) of Nedd4-2 expression in whole-cell lysates from Nav1.5-HEK cells or NRCMs cells treated either with ionomycin (IM) or thapsigargin (Thap) for 6 h, n=3, * P <0.05 vs. Ctrl. (c), mRNA level of Nedd4-2 (normalized to GAPDH) in NRCMs treated with IM or Thap, n=3, * P <0.05 vs. Ctrl. HEK 293 cells transfected by Nav1.5 together with pcDNA3, HA-tagged Nedd4-2 or HA-tagged Nedd4-2-CS were treated without (Ctrl) or with IM in the presence of MG132 (proteasome inhibitor) for Co-IP assay presented in (d), (e) and (f). (d), The whole-cell lysates were immunoprecipitated (IP) with anti-Nav1.5 and immunoblotted (IB) with anti-Nav1.5 and/or anti-Nedd4-2 antibody respectively to detect the bound Nedd4-2 by Nav1.5. The densitometric measurements (Nedd4-2/Nav1.5 ratio) were presented below the representative blots (n=3, * P <0.05 vs. Ctrl, # P <0.05 vs. Nedd4-2). (e) The cell lysates were immunoprecipitated by anti-HA antibody and immunoblotted against Nav1.5 and/or Nedd4-2 and the densitometric measurements (Nav1.5/Nedd4-2 ratio) were presented below the representative blots (n=3, * P <0.05 vs. Ctrl, # P <0.05 vs. Nedd4-2). (f) The cell lysates were immunoprecipitated by anti-ubiquitin and the precipitates were immunoblotted using anti-Nav1.5 to detect its ubiquitination level, the densitometric measurements (normalized to IgG) were presented below the blots (n=3, * P <0.05 vs. Ctrl, # P <0.05 vs. Nedd4-2). The input proteins of cell lysates were loaded in a separate gel and the blot indicates the same amounts of starting materials used for Co-IP.

Figure 5 Calcium downregulates Nav1.5 via Nedd4-2 pathway. (a), Representative immunoblot and (b) densitometric measurements (normalized to actin) of Nav1.5 in Nav1.5-HEK cells transfected with pcDNA3, Nedd4-2 or Nedd4-2 CS and treated without (Control, Ctrl) or with IM (1 μ M, 6 h) as indicated. n=3, * P <0.05 vs. pcDNA3, # P <0.05 vs. Nedd4-2. Y1977A-Nav1.5 or WT-Nav1.5 transiently transfected HEK 293 cells were treated with IM for 24 h. The representative immunoblot (c) and (d) densitometric measurements (normalized to actin) of Nav1.5 expression, n=3, * P <0.05 vs. WT. Y1977A-Nav1.5 was co-transfected with Nedd4-2 in HEK 293 cells and treated with IM. The representative immunoblot (e) and (f) densitometric measurements (normalized to actin) of Nav1.5 expression, n=3.

Figure 6 The expression and co-localization of Nav1.5 and Nedd4-2 or ubiquitin in HF rats. Representative immunoblots (a) and (b) densitometric measurements (normalized to actin) of Nav1.5 and Nedd4-2 in the SO and HF rat left ventricle tissue. n=6 for each group, * P <0.05 vs. SO, † P <0.01 vs. SO. (c) The relative SCN5A and Nedd4-2 mRNA level (normalized to GAPDH) in SO and HF rat left ventricle tissue. n=6 for each group, * P <0.05 vs. SO. The confocal images of isolated adult rat cardiomyocytes from SO and HF groups which were double-immunolabeled with anti-Nav1.5 and Nedd4-2 antibodies (d) or with anti-Nav1.5 and ubiquitin antibodies (e).

Figure 7 BAPTA-AM rescued the reduced Nav1.5 or increased Nedd4-2 in hypertrophied NRCMs induced by isoproterenol (ISO) or angiotensin II (AngII) treatment. NRCMs were treated with ISO (10 μ M) or AngII (1 μ M) for 24 h, then together without or with BAPTA-AM (BAP, 5 μ M) for another 24

h. (a), Representative immunoblot and (b) densitometric measurements (normalized to actin) of Nav1.5 and Nedd4-2 in NRCMs. n=3, $\dagger P < 0.01$ vs. Ctrl, $\# P < 0.05$ vs. ISO or AngII. (c), Typical I_{Na} current traces in NRCMs treated without ISO (Ctrl), with 10 μ M ISO (ISO), or 10 μ M ISO plus BAPTA-AM (ISO+BAP). (d), The summarized peak I_{Na} density, n=12~14 cells, $\dagger P < 0.01$ vs. Ctrl, $\# P < 0.05$ vs. ISO. (e), The I/V curve of the summarized I_{Na} in NRCMs cells treated with ISO without or with BAP. (f), Steady-state activation and inactivation curves of I_{Na} . The data were obtained and analyzed using the same protocols used in Figure 1.

Figure 8 Proposed scheme illustrating that calcium reduces Nav1.5 by increasing Nedd4-2, a possible mechanism in HF. Increased Ca^{2+} “leakage” through malfunctioned RyR2 channel from sarcoplasmic reticulum (SR), increased Ca^{2+} entry from extracellular space via L-type calcium channel (LTCC) and reduced SR Ca^{2+} uptake by sarcoplasmic reticulum Ca^{2+} ATPase (SERCA) elevated cytosolic Ca^{2+} level, and enhances Nedd4-2 expression and function. The WW domain of Nedd4-2 binds to the PY motif of Nav1.5 to initiate Nedd4-2-mediated ubiquitination and internalization which may be either degraded or recycled back to the membrane.















